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14. ABSTRACT The purpose of this grant was to develop RNA vectors capable of delivering functional RNAi.					
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a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 212-241-7849

Report Title

Final Report: Research Area 14.3 Microbiology and Biodegradation

ABSTRACT

The purpose of this grant was to develop RNA vectors capable of delivering functional RNAi.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
08/03/2012	1.00 J. S. Shapiro, R. A. Langlois, A. M. Pham, B. R. tenOever. Evidence for a cytoplasmic microprocessor of pri-miRNAs, RNA, (05 2012): 0. doi: 10.1261/rna.032268.112
08/03/2012	2.00 Ryan A Langlois, Jillian S Shapiro, Alissa M Pham, Benjamin R tenOever. In Vivo Delivery of Cytoplasmic RNA Virus-derived miRNAs, Molecular Therapy, (11 2011): 0. doi: 10.1038/mt.2011.244
08/07/2014	6.00 S. Schmid, L. C. Zony, B. R. tenOever. A Versatile RNA Vector for Delivery of Coding and Noncoding RNAs, Journal of Virology, (12 2013): 0. doi: 10.1128/JVI.03267-13
08/13/2013	3.00 Ryan A Langlois, Randy A Albrecht, Brian Kimble, Troy Sutton, Jillian S Shapiro, Courtney Finch, Matthew Angel, Mark A Chua, Ana Silvia Gonzalez-Reiche, Kemin Xu, Daniel Perez, Adolfo García-Sastre, Benjamin R tenOever. MicroRNA-based strategy to mitigate the risk of gain-of-function influenza studies, Nature Biotechnology, (08 2013): 0. doi: 10.1038/nbt.2666
08/13/2013	4.00 Jillian S. Shapiro, Simone Backes, Leah R. Sabin, Alissa M. Pham, Ismarc Reyes, Bernard Moss, Sara Cherry, Benjamin R. tenOever. Degradation of Host MicroRNAs by Poxvirus Poly(A) Polymerase Reveals Terminal RNA Methylation as a Protective Antiviral Mechanism, Cell Host and Microbe, (08 2012): 0. doi: 10.1016/j.chom.2012.05.019
TOTAL:	5

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received

Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received

Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received

Paper

TOTAL:

(d) Manuscripts

Received Paper

08/13/2013 5.00 Andrew Varble, Asiel Benitez, Sonja Schmid, David Sachs, Jaehee Shim, Ruth Rodriguez-Barrueco, Maryline Panis, Marshall Crumiller, Jose M. Silva, Ravi Sachidanandam, Benjamin R. tenOever. RNAi screening in vivo identifies host determinants of virus replication, Cell Host & Microbe (10 2013)

TOTAL: 1

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Recombinant microRNA containing RNA Viruses and Uses Thereof, US Patent # 61/351,908.

Patents Awarded

Our patent application is still pending

Awards

American Society of Virology Young Investigator Award.
Fulbright Award – Tocqueville Distinguished Chair Award.

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Jillian Shapiro	0.25	
Chait Zoni	0.25	
FTE Equivalent:	0.50	
Total Number:	2	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
Sonja Schmid	0.50
FTE Equivalent:	0.50
Total Number:	1

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Benjamin tenOever	0.10	
FTE Equivalent:	0.10	
Total Number:	1	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees⁷

<u>NAME</u>

Total Number:

Names of personnel receiving PHDs

<u>NAME</u>

Jillian Shapiro

Total Number:	1
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Names of other research staff

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
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FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

5 Recombinant microRNA containing RNA Viruses and Uses Thereof

Patent Filed in US? (5d-1) Y

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) Y

Foreign Countries of application (5g-2):

5a: Benjamin tenOever

5f-1a: Icahn School of Medicine

5f-c: 1468 Madison Avenue

New York

NY 10029

Scientific Progress

This grant, which has now expired, was used to develop novel RNA virus-based vectors for the purposes of delivering small interfering RNAs to cells both in vitro and in vivo.

Technology Transfer

One patent was filed based on this grant in June 2010. It was previously described in a corresponding progress report.

Title: Final Report: Research Area 14.3 Microbiology and Biodegradation

Subtitle: Development of RNA-based vectors for *in vivo* delivery of siRNAs

Summary: This grant focused on the development of an innovative technology, which can silence multiple gene products *in vivo*, and thus provides the ability to treat various disease states in a rapid and cost effective manner. The basis of this work is rooted in a recent development from our lab demonstrating that cytoplasmic RNA-based vectors can be engineered to confer functional RNA interference *in vivo*. This proposal aims to expand on these finding by generating both replication competent and incompetent vectors, capable of inducing robust expression of artificial miRNAs (amiRNAs, siRNAs designed to conform to a miRNA structure).

Proposed Statement of Work: In an effort to develop a novel platform to produce small intracellular RNAs of interest we proposed to engineer RNA-based viral vectors encoding amiRNAs. Our approach combines the specificity of RNA interference (RNAi) with synthetic biology to build virus-inspired vectors capable of delivering a small RNA payload engineered to specifically target an mRNA of interest. This proposal is unique from past attempts to exploit RNAi as it utilizes RNA-based virus-like vectors (VLVs) which do not persist or cause cytopathic effects and it utilizes these vehicles in the context of a novel class of small RNA processing. This proposal was originally divided into three specific tasks - the breakdown of which are listed below.

Task 1. The generation of artificial miRNAs: This task aimed to develop a model hairpin that can be processed by the cell's endogenous machinery to generate an engineered small RNA of interest. We identified miR-124 as an ideal candidate.

Task 2. Engineering replication-incompetent small RNA delivery platforms: This task was designed to expand on our first-generation of viruses, which were capable of delivering functional RNA hairpins, and adapt these microbes to replication-incompetent VLVs. We found Influenza- and Sindbis-based vectors were the most effective and versatile.

Task 3. Determining efficacy and mechanism of action: Here we proposed to determine whether delivery of an artificial miRNA is a viable silencing strategy and ascertain the cellular mechanism by which it occurs. Here, we implicated the cytoplasmic translocation of Drosha in this processing activity and determined that VLVs could be engineered to elicit potent sequence-specific silencing.

Deliverables: This grant resulted in significant publications and advancements concerning each of the tasks proposed. Below is a summary of the results for each task.

Task 1: The generation of artificial microRNAs. The folding of a miRNA generally includes a 60 nucleotide pre-miRNA hairpin composed of a 19-25nt double-stranded stem connected by 5-10 nt, single-stranded bulge (Bartel, 2004). While this structure is essential for recognition of the microprocessor, additional 5' and 3' sequences of the pri-miRNA are also important (Bartel, 2004). Processing of the hairpin results in the generation of an RNA duplex which originates from the stem of the hairpin and is ultimately responsible for RISC-mediated PTS. Given the knowledge of miRNA topography, a number of studies have successfully generated artificial miRNAs in which the stem of a prototypic miRNA is replaced with a nt sequence of interest (Zeng et al., 2002). In fact, this work allows one to generate an artificial miRNA that is perfectly complementary to a given transcript, thereby transforming it to an siRNA. This technology has not only been applied to generate miRNAs against

specific pathogens (Israsena et al., 2009; Zeng et al., 2002), but has been utilized to generate whole-genome targeting libraries (Shtutman et al., 2010; Silva et al., 2005). Therefore, task one of this proposal was to simply adapt this technology to our RNA-based delivery platforms which initially required determining what miRNAs were best suited for siRNA construction.

Determining the versatility of artificial microRNAs

For the purposed of harnessing the silencing potential of small RNAs, we compared the versatility of two distinct miRNA hairpins, namely miR-124 and miR-30 (Figure One). We designed both hairpins to generate the same siRNA against GFP (depicted in green) and used overexpression assays to measure silencing potential. These results clearly demonstrated that miR-124 processing resulting in increased silencing potential. As a result, we used miR-124 as our miRNA scaffold for all hairpin designs.

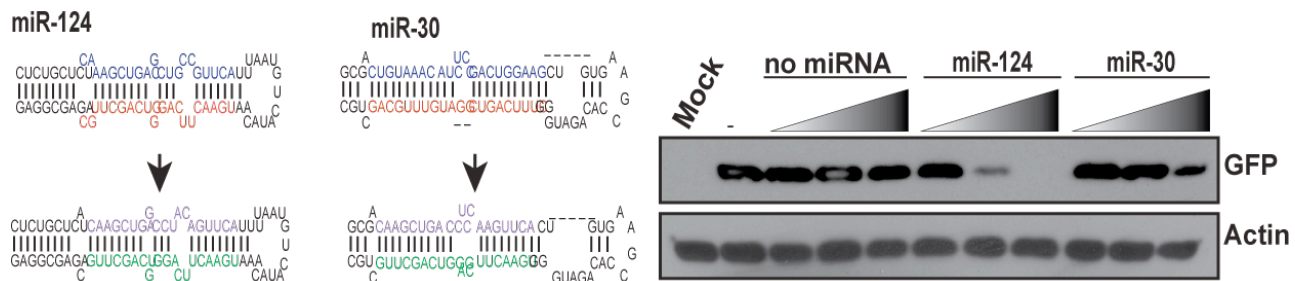


Figure One. Left. Models depicting microRNA 124 (miR-124) and miR-30, both designed to generate a small interfering RNA against GFP. Right. Western blot of whole cell extract derived from cells co-expressing miR-124 or miR-30 targeting GFP in the presence of GFP.

In an effort to further evaluate the silencing versatility of amiRNAs based on miR-124, we generated five independent amiRNAs against GFP using the same miR-124 scaffold (Figure Two). These data demonstrated that, regardless of siRNA sequence, each of the five miRNAs processed efficiently and 4 of the 5 demonstrated effective silencing.

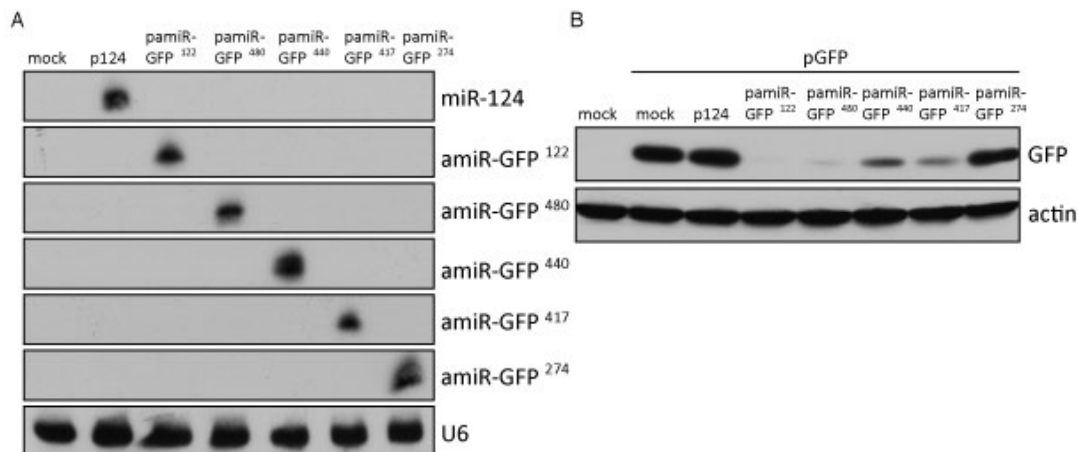


Figure Two (A) Northern blot of VLV based amiRNAs targeting various positions of GFP mRNA. (B) Western blot of VLV-based silencing corresponding to the amiRNAs depicted in (A).

Task 2. Engineering replication-incompetent small RNA delivery platforms: This task involved determining whether all RNA virus-based vectors were capable of generating siRNAs in the context of a miR-124 scaffold (Based on Task 1) and whether delivery and silencing could be achieved *in vitro*. These efforts resulted in three publications and generated many of the reagents that were used in the publications for task three. These papers include:

Shapiro, J.S., Langlois, R.A., Pham, A.M. and B. R. tenOever. Evidence for a cytoplasmic microprocessor of pri-miRNAs. *RNA*. 2012, Jul;18(7): 1338-46. PMID: 22635403

Langlois, R.A., Shapiro, J.S., Pham, A.M. and B. R. tenOever. *in vivo* delivery of cytoplasmic RNA virus-derived miRNAs. *Mol. Ther.* 2011, Nov. 15,(20)(2):367-75. PMID: 22086233

Langlois, R.A., Albrecht, R., Kimble, B., Sutton, T., Shapiro, J., Finch, C., Angel, M., Chua, M.A. Gonzalez-Reiche, A., Xu, K., Perez, D., García-Sastre, A. and B.R. tenOever. Molecular risk mitigation of gain-of-function studies with influenza viruses. *Nature Biotechnology*. 2013, Aug, 31, 844-847. PMID: 23934176

Schmid, S. Zony, X., and B.R. tenOever. A versatile RNA vector for delivery of coding and non-coding RNAs. *Journal of Virology*. 2014, 88(4), 2333-6. PMID: 24307584

Hairpin capacity of nuclear-RNA-based vectors. The discovery that RNA viruses, lacking any DNA intermediate, could be engineered to express both coding and non-coding RNAs, suggests that this platform may have therapeutic value as a delivery vehicle. Although no RNA virus, void of a DNA intermediate, has been found to encode a canonical miRNA, we, and others, have shown that RNA viruses can be engineered to generate functional small RNAs *in vivo* (tenOever, 2013). Here, we explored the potential of replication-incompetent RNA virus-like vectors (VLVs) for the production of small RNAs. We show that influenza A virus (IAV), Sindbis (SINV), and Vesicular Stomatitis Virus (VSV) -derived VLVs can be engineered to deliver functional small RNAs in primary cells and *in vivo*, leading to efficient target gene knock-down.

To generate replication-incompetent influenza-based VLVs capable of eliciting RNAi, we used a previously published system for production of IAV-based VLVs not encoding the viral glycoprotein hemagglutinin (HA) (tenOever, 2013). All constructs used for VLV production were based on the mouse-adapted IAV strain A/Puerto Rico/8/1934(H1N1). We replaced the open reading frame (ORF) of

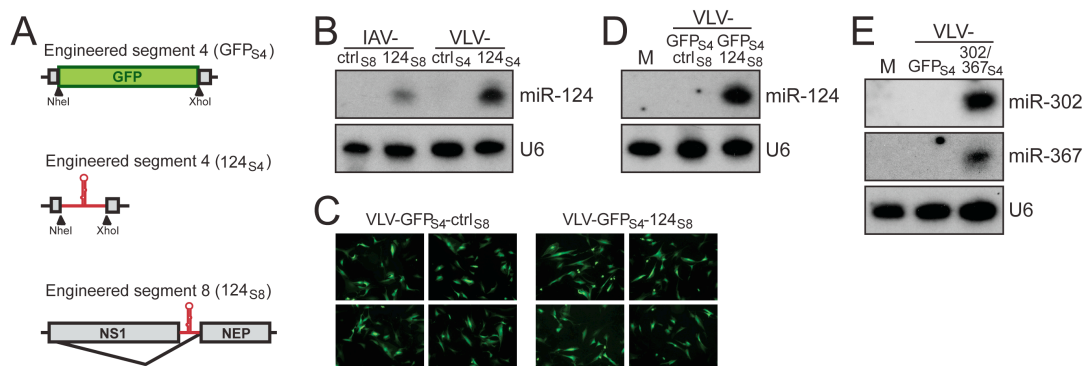


Figure Three. (A) Schematic depicting engineered segments four (S4) and eight (S8) of influenza A virus (IAV) encoding green fluorescent protein (GFP) or containing a microRNA (red). S8 encodes the miRNA from an intergenic region between the non-structural protein 1 (NS1) and the nuclear export protein (NEP). S4 expressing GFP flanked by the packaging sequence of influenza A HA (depicted by grey boxes). To generate S4 coding for a small RNA, GFP was replaced with a primary miRNA using NheI and XhoI. (B) Small RNA northern blot of fibroblasts cells treated with IAV-124_{S8} or VLV-124_{S4} at a multiplicity of infection (MOI) of three and analyzed at one day post treatment (dpt). U6 was used as a loading control. (C) and (D) Fibroblasts were treated with VLV-GFP_{S4}-ctrl_{S8} or VLV-GFP_{S4}-124_{S8} at MOI three and expression of GFP (C) or miR-124 (D) was analyzed by fluorescence microscopy or small RNA northern one dpt, respectively. (E) Small RNA northern blot probed for miR-302, miR-367, and U6 upon VLV-302/367_{S4} treatment as described in (D).

segment four, with either a scrambled RNA (ctrl_{S4}) or the primary *Mus musculus* miR-124-2 transcript (124_{S4}) (Fig. 3A). We produced and propagated the respective VLVs on Madin-Darby canine kidney (MDCK) cells stably expressing HA (HA-MDCK). HA-MDCK cells were also utilized to determine VLV titres by standard plaque assay. First we compared miR-124 expression between replication competent IAV expressing miR-124 (described in (tenOever, 2013) and referred to as IAV-124_{S8} herein) and the replication incompetent vector, VLV-124_{S4}.

Surprisingly, normal human dermal fibroblasts treated with VLV-124_{S4} exhibited a 3-fold higher expression of miR-124 than cells treated with IAV-124_{S8}, as shown by small RNA northern (Fig. 3B). Encouraged by this result, we next aimed to explore the maximum expression capacity of the VLVs. In an effort to produce both coding and non-coding RNAs from a single VLV, we utilized a segment four expressing green fluorescent protein (GFP) (Fig.3A) in conjunction with segment eight expressing scrambled RNA or miR-124 (herein referred to as VLV-GFP_{S4}-ctrl_{S8} or VLV-GFP_{S4}-124_{S8}). Fibroblasts treated with either VLV showed high expression of GFP at one day post treatment (dpt) (Fig. 3C). Furthermore, fibroblasts treated with VLV-GFP_{S4}-124_{S8} expressed high levels of miR-124 (Fig. 3D), demonstrating that VLVs can simultaneously deliver both coding and non-coding RNAs into primary human cells.

We next determined whether VLVs could express multiple non-coding RNAs. To this end we cloned the miR-302/367 cluster into segment four. This miRNA cluster contains five tandem hairpins for miR-302b, miR-302c, miR-302a, miR-302d and miR-367 respectively (302/367_{S4}). Following rescue of the influenza virus-based particles, fibroblasts cells were treated to assess small RNA levels. VLV-302/367_{S4} expressed high levels of miR-302 and miR-367 (Fig. 3E). While northern blot analysis could not distinguish between 302a-d, it is noteworthy that miR-302 and miR-367 could be detected. Given these miRNAs represent the 5' and 3' ends of the non-coding RNA, these results suggest that the five miRNAs were processed fully when encoded within the VLV. Taken together, these experiments suggest VLVs can be engineered to deliver multiple coding and non-coding RNAs.

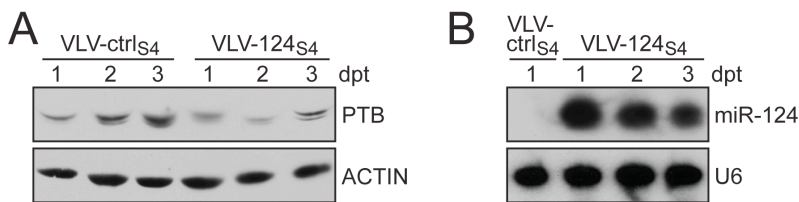


Figure Four. (A) and (B) Fibroblasts were treated with VLV-ctrl_{S4} or VLV-124_{S4} at an MOI of three and expression of the endogenous miR-124 target polypyrimidine tract binding protein (PTB) (A) as well as miR-124 (B) was analyzed by western or northern blot, respectively, at one, two, and three dpt. ACTIN or U6 served as loading controls.

We next determined whether VLV-delivered miR-124 is functional. To this end we treated fibroblasts with VLV-124_{S4} and analyzed the expression levels of the endogenous miR-124 target, polypyrimidine tract binding protein (PTB), by western blot (WB). Following treatment with VLV-124_{S4}, PTB levels were reduced by 75% and 60% at two and three dpt, respectively (Fig. 4A). Consistent with this result, miR-124 was robustly expressed at one, two, and three dpt (Fig. 4B). Deep sequencing of IAV-

based small RNA expression systems have suggested that the vector can generate ~50000 copies of a miRNA per cell in less than 6hrs. Given that this level of expression exceeds the majority of cellular miRNAs, this delivery system may result in unwanted cytotoxicity. As such, we next wanted to determine if production of exogenous RNAs could be modulated in the context of the VLV. To this end, we utilized a previously described strategy that relies on the expression of host miRNAs to limit virus infection. For this approach, the viral genome of interest is engineered to contain one or more perfect complementary target sites for a host miRNA, resulting in cleavage of the viral target site-containing transcript by the host RNAi machinery. This ultimately leads to attenuation of viral replication when an essential gene is silenced.

Hairpin capacity of cytoplasmic-RNA-based vectors. Given the success of IAV based delivery systems, we next determined whether we could adapt this RNA silencing platform to a cytoplasmic RNA based vector. To this end, we inserted miR-124, miR-122, or both miR-124 and miR-122 into a self-replicating SINV-based RNA platform. These RNAs were found to readily amplify in cell culture and generate high levels of all encoded miRNAs (Fig. 5). This delivery technology could also be demonstrated with cytoplasmic RNA vectors of negative polarity, exemplified by VSV below (Figure 6). This experiment directly compares miR-124 synthesis from three different RNA vectors in the context of an *in vivo* delivery system.

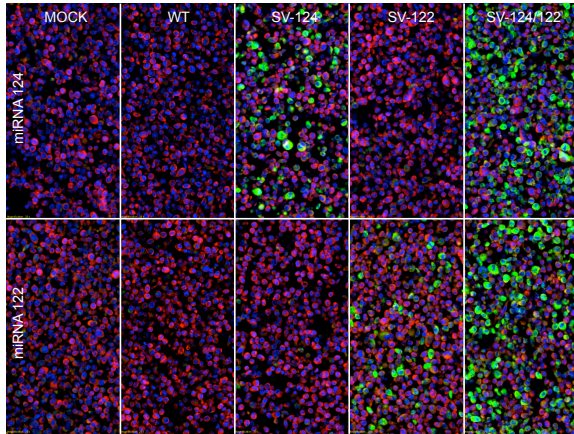


Figure Five. RNA in situ hybridization demonstrating *in vitro* production of microRNAs from a self-replicating SINV-based RNA construct.

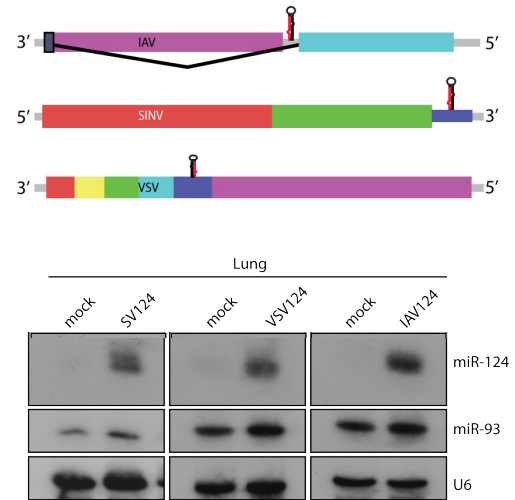


Figure Six. Top. Cartoon schematic of self-replicating RNAs engineering to synthesize miR-124. Constructs based on influenza A virus (IAV), Sindbis virus (SINV), and Vesicular Stomatitis Virus (VSV). Bottom. Northern blot derived from whole lung of mice administered with the vectors depicted.

Silencing potential of nuclear-RNA-based vectors. To test whether the nuclear RNA-based VLVs would allow target gene knock-down in hematopoietic cells. We chose bone marrow derived macrophages (BMM), since resident macrophages in the lung would be likely to take up VLVs upon intranasal treatment. Small RNA northern blot showed expression of miR-124 one dpt with VLV-124_{S4} (Fig. 7A). Furthermore, *GFP* transcripts were reduced by approximately 40% at one dpt with VLV-amiR-GFP_{S4} (Fig. 7B). To analyze GFP protein expression, we stained BMMs treated with either VLV-ctrl_{S4} or VLV-amiR-GFP_{S4} with an antibody detecting NP and performed FACS analysis. As shown in Fig. 7C, treatment with VLV-amiR-GFP_{S4} reduced expression of GFP in NP⁺ cells at three dpt. Finally we tested whether the VLVs could deliver a small RNA *in vivo*. To this end we treated wildtype mice intranasally with VLV-124_{S4}. We could detect robust levels of miR-124 in whole lungs at one dpt (Fig. 7D) suggesting this system may be means of respiratory tract delivery for siRNAs.

These results show here for the first time that replication-incompetent nuclear-RNA based VLVs lacking a DNA intermediate can be used to deliver small RNAs. Target gene knock-down in primary human and murine fibroblasts as well as hematopoietic cells could be observed for at least three dpt. Furthermore we found that miRNA-mediated silencing of the vector represents a strategy that permits one to modulate small RNA production and eliminate cellular toxicity while also defining tissue specificity if desired. This finding later found a unique application as a means of molecular biocontainment (Langlois et al, 2013). In addition, these VLVs can be engineered to simultaneously express both coding and non-coding RNAs of interest.

Taken together, we show that nuclear RNA virus-based VLVs are versatile vectors that could be utilized for a range of genetic applications. Vectors based on RNA viruses that lack a DNA intermediate possess a number of desirable attributes (tenOever, 2013). In contrast to vectors based on DNA viruses or lentiviruses, VLVs do not integrate into the host genome and would therefore not perturb endogenous gene expression. Furthermore, these vectors appear to bypass many of the cellular toxicities that have thus far been documented for stably integrating vectors as long-term exogenous expression of small RNAs saturates the endogenous RNAi pathway. Furthermore, IAV vectors with limited replicative capacity have already been FDA approved suggesting this platform to be safe. In all, this current study provides an alternative methodology for the delivery of both coding and non-coding RNAs and demonstrates the versatility of this vector-based delivery platform for future utilities.

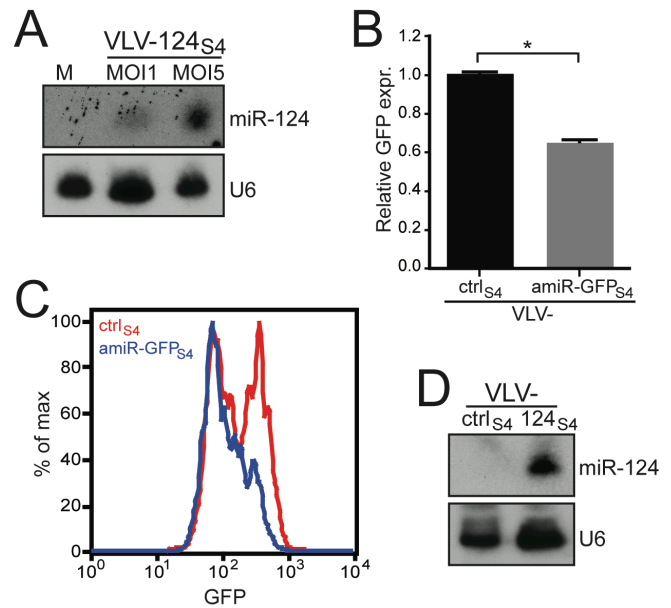


Figure Seven. (A) GFP^{+/+} BMMs were treated at two different MOIs with VLV-124_{S4} and expression of miR-124 was analyzed by small RNA northern at one dpt. (B) GFP^{+/+} BMMs were treated at an MOI of five with VLV-ctrl_{S4} or VLV-amiR-GFP_{S4}. One dpt expression levels of *GFP* transcripts were analyzed by standard qPCR. (C) GFP^{+/+} BMMs were treated at an MOI of five with VLV-ctrl_{S4} or VLV-amiR-GFP_{S4}. Three dpt expression of *GFP* protein was analyzed by FACS analysis using intracellular staining with a NP-specific antibody. (D) C57BL/6 mice were intranasally VLV-ctrl_{S4} or VLV-124_{S4}. RNA from whole lungs was isolated and expression of miR-124 was analyzed by small RNA northern.

Silencing potential of cytoplasmic-RNA-based vectors. To demonstrate the capacity of SINV to act as a platform for amiRNA delivery we designed a collection of five unique hairpins targeting enhanced green fluorescent protein (GFP). Accurate processing and PTS of these amiRNAs was confirmed by small RNA northern blot and western blot, respectively (Not shown). These data demonstrate four of the five hairpins effective in silencing GFP; with two hairpins showing complete knockdown (Task 1). To ascertain whether incorporation of a GFP amiRNA conferred silencing activity onto SINV, amiRNA-GFP¹²², which was designed to conform to the structure of miR-124 (Task 1), was inserted into the recombinant SINV. As previously described (Shapiro et al., 2012; Shapiro et al., 2010), despite the cytoplasmic replication of SINV, amiRNA-GFP¹²² demonstrated accurate processing and robust expression at every time point evaluated following SINV-amiR-GFP infection (Figure 8A). More importantly, the capacity to generate the amiRNA also enabled the virus to silence GFP expression in the context of infection (Figure 8B).

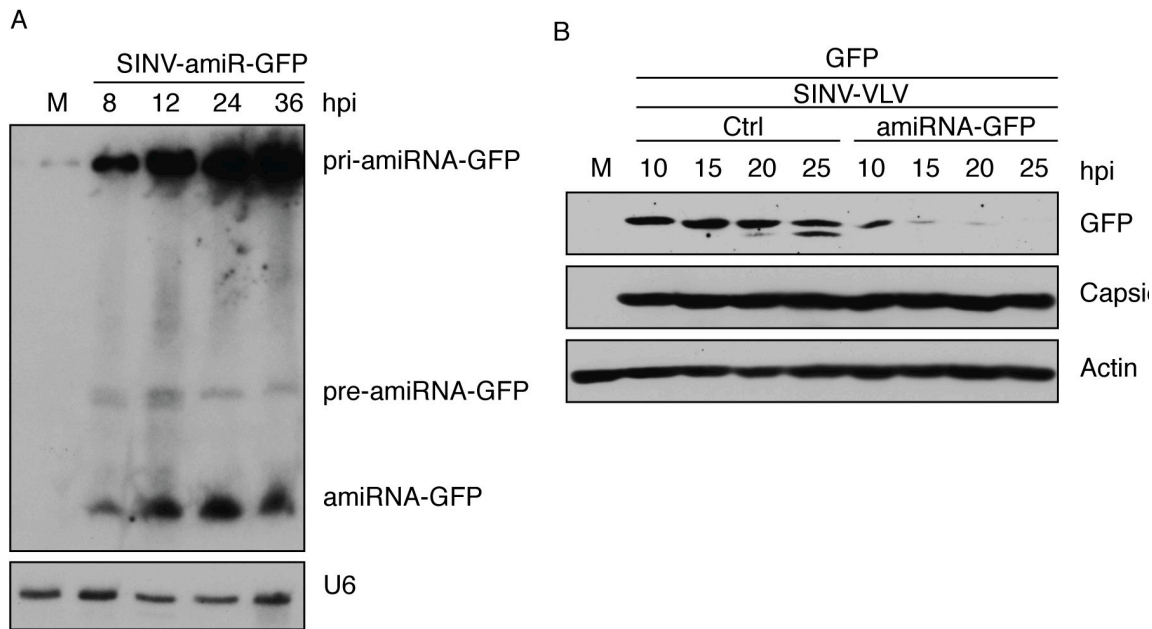


Figure 8. (A) Northern blot of a SINV-based VLV designed to synthesize an siRNA against GFP. (B) Western blot of fibroblasts treated with the SINV vector in (A) demonstrating the capacity to silence GFP.

Task 3. Determining efficacy and mechanism of action: Having demonstrated the capacity of RNA-based VLVs to generate siRNAs, we next sought to (I) determine how this process was occurring given the non-canonical nature of cytoplasmic processing and (II) ascertain the potential of this delivery technology in effectively silencing targets *in vivo*. This task resulted in the following publications.

Backes, S., Shapiro, J.S., Sabin, L.R., Pham, A.M. Reyes, I., Moss, B., Cherry*, S. and B.R. tenOever*. Degradation of host microRNAs by a viral poly(A) polymerase reveals terminal 2'-O-methylation as a protective anti-viral mechanism. *Cell Host and Microbe*. 2012, Aug;12(2): 200-210. PMID: 22901540

Varble, A., Benitez, A., Schmid, S., Sachs, D., Shim, J.V., Rodriguez-Barrueco, R., Panis, M., Crumiller, M., Silva, J., Sachidanandam, R. and B.R. tenOever. RNAi screening *in vivo* identifies critical components to the antiviral response. *Cell Host & Microbe*. 2013, 14, 346-356. PMID: 24034620

Shapiro, J.S., Schmid, S., Aguado, L., Sabin, L.R., Cherry, S. and B.R. tenOever. Drosha is an interferon-independent antiviral factor. *PNAS*. 2014. 111(19): 7108-7113. PMID: 24778219

I. Mechanism of action. In an effort to determine whether the core components of the nuclear microprocessor were required for c-pri-miRNA cleavage, despite the disparity in cellular localization, we characterized rSINV124-derived miR-124 synthesis in DGCR8- and Drosha-deficient cells (Fig. 9). As these fibroblast-derived cell lines are conditional knockouts, cells were initially treated with replication-deficient Adenovirus vectors expressing either green fluorescent protein (GFP) or a GFP-Cre recombinase fusion protein (GFP and Cre, respectively). Six days post-treatment of *Dgcr8^{fl/fl}* and *Rnasen^{fl/fl}* (the gene encoding Drosha) cells, we could confirm complete loss of endogenous, canonical miR-93 (which normally maintains concentrations of 10,000 copies/cell (Tye 1999)) (Fig. 9). Following confirmation of DGCR8 and Drosha disruption, we infected with rSINV124 for 16 hrs at an MOI of five and analyzed miR-124 synthesis (Fig. 9). Interestingly, despite the discrepancy between the cytoplasmic localization of the c-pri-miRNA and the nuclear localization of the canonical microprocessor, loss of Drosha resulted in complete ablation of virus-derived pre- and mature-miR-124 (Fig. 9, lanes 4-5).

Surprisingly, in contrast to Drosha-dependency, loss of DGCR8 only altered the accumulation of mature miR-124 while having no impact on the conversion of c-pri-miRNA to pre-miRNA, making its involvement in cytoplasmic processing difficult to discern (Fig. 9, lanes 6-7). The selective decrease in mature miRNA suggests that while DGCR8 is not required for Drosha-mediated cleavage, it may be essential for its accuracy. In support of this hypothesis, loss of DGCR8 does result in an accumulation of pre-miR-124 suggesting this RNA may no longer be an optimal Dicer substrate. Alternatively, the cytoplasmic microprocessor may be DGCR8-independent and the phenotype due to an indirect effect on the cell's small RNA machinery following the loss of endogenous miRNAs. Future work will be needed to discern between these two possibilities.

In an effort to determine whether any of the other known miRNA biogenesis components were required for c-pri-miRNA processing, we additionally infected cells lacking Dicer, TRBP2, or PACT (encoded by *Dicer1*, *Tarbp*, and *Prkra* genes, respectively) with rSINV124 (Fig.9, lanes 8-13). Northern blot analysis from rSINV124-infected *Dicer1* knockout cells demonstrated abundant levels of c-pri-miR-124 and the ~60nt pre-miRNA, with only mature miR-124 absent (Fig.9, lane 9). The levels and size of pre-miR-124 were comparable to that produced in rSINV124-infected WT cells, suggesting that Dicer is not involved in cleavage of the c-pri-miRNA into pre-miRNA. In the absence of TRBP2 and of PACT, the levels of all three miRNA species (c-pri-, pre-, and mature) remained unchanged (Fig. 9, lanes 10-1). Given the shared 42% amino acid homology between TRBP2 and PACT (Haase et al. 2005), these results suggest these dsRNA binding proteins are not essential, or are functionally redundant, with regards to Drosha- and/or Dicer-mediated processing of c-pri-miRNAs.

To conclude our genetic characterization of c-pri-miRNA processing, we investigated a requirement for AGO2 (encoded by the *Eif2c2* gene). Given previous results demonstrating the role of AGO2 in the generation of miR-451 (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang et al. 2010), we investigated whether AGO2 had a role in processing, despite the fact that miR-124 does not conform to the structural requirements for AGO2-dependent cleavage (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang et al. 2010). To this end, we infected cells deficient in AGO2 with rSINV124 at an MOI of one for 16 hrs to compare virus-derived cytoplasmic miR-124 synthesis to that of infected WT cells. These data demonstrated no alteration in pre- or mature-miR-124 production (Fig.9, lane 15). Taken together, these results implicate the RNase III proteins, Dicer and Drosha, and possibly the dsRNA binding protein DGCR8, in the accurate processing of c-pri-miRNAs.

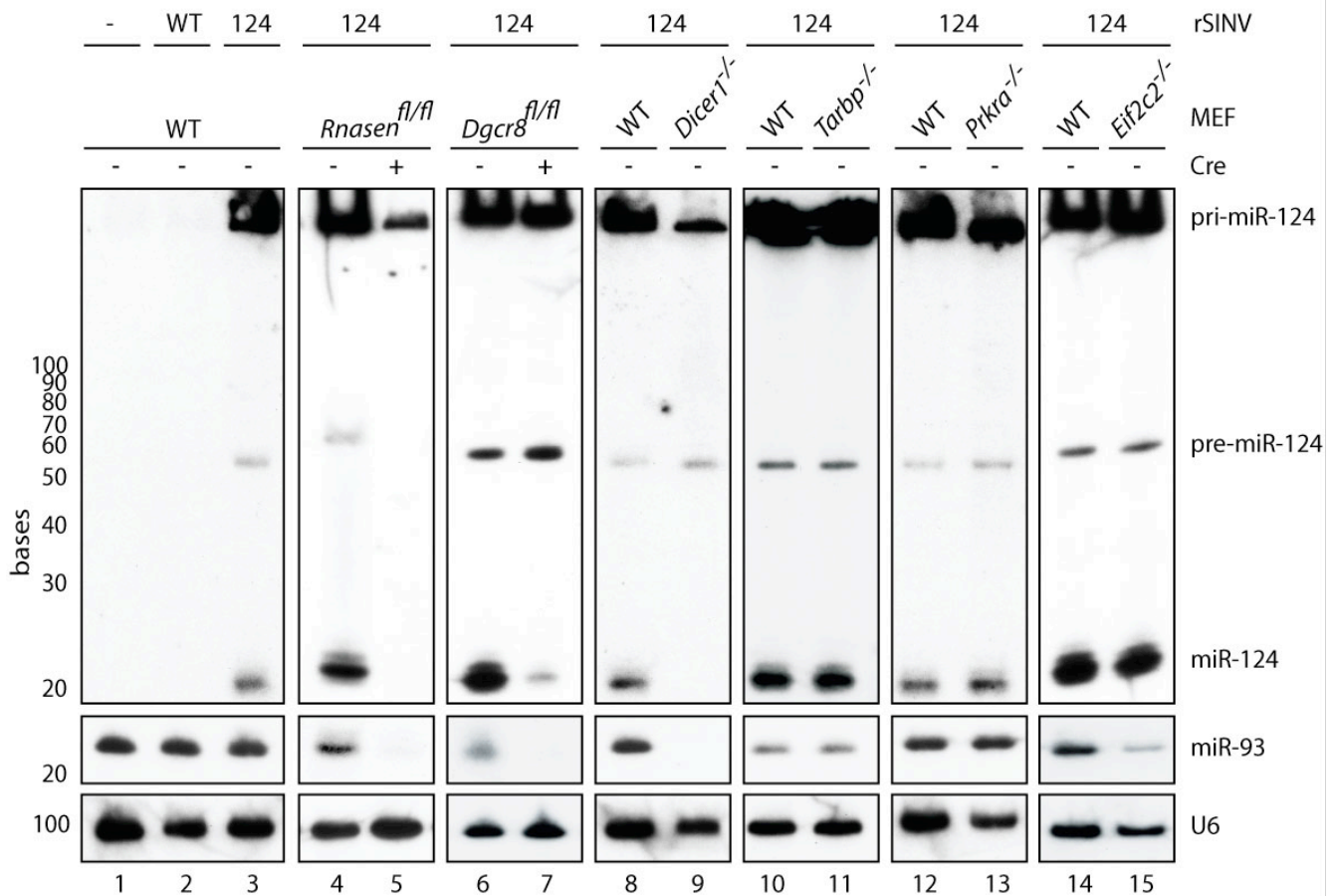


Figure Nine. Cytoplasmic requirements for processing of pri-miRNAs. Northern blot depicting WT MEFs or MEFs deficient in Drosha, DGCR8, Dicer, TRBP2, PACT, or AGO2 (*Rnasen*, *Dgcr8*, *Dicer1*, *Tarbp*, *Prkra*, or *Eif2c2*, respectively) mock treated or infected with WT-rSINV or rSINV124 (MOI=5 for Drosha and DGCR8 deficient cells; MOI=1 for Dicer, TRBP2, PACT and AGO2 deficient cells; 16 hrs for all samples) and RNA was analyzed via small RNA northern blot probed for miR-124 (top), miR-93 (middle), and U6 (bottom).

II. Efficacy and application. Having characterized the mechanism by which VLV-derived siRNAs are processed, we wished to complete the studies of this grant proposal by applying this technology to in vivo applications. These studies, some of which are still ongoing, have resulted in some remarkable discoveries and unanticipated applications. For example, the application of miRNAs in cytoplasmic DNA viruses resulted in the discovery that poxviruses destroy all miRNAs through the expression of an antagonistic protein called VP55 (Backes et al. 2012). In addition to this, we also developed the original proposed task of utilizing the hairpins designed in task 1 with the vectors in task 2 to perform in vivo targeting experiments.

Given the replicative advantage that could be conferred onto a VLV based vectir through use of an encoded amiRNA, we utilized a library of viruses containing unique hairpins to determine whether natural selection would parse out advantageous non-coding RNAs in the context of an *in vivo* infection and ascertain the timing of enrichment. This initial SINV library was composed of approximately 200000 unique hairpins, each designed to target a murine ORF (Silva et al., 2005). Population infection experiments, followed by small RNA cloning and deep sequencing analysis, demonstrated approximately 75% of the amiRNAs were processed as predicted (Not shown). This viral population was subsequently administered into mice via footpad injection and virus was isolated from total spleen 48 hrs post infection. Composition of amiRNAs present in viral populations were monitored through deep sequencing and revealed a clear enrichment of certain hairpins by the third mouse passage (Fig. 10A). To

determine if this enrichment was the direct result of the hairpin or a consequence of unrelated mutations elsewhere in the viral genome, we employed a genetic reset between passages three and four. The reset was accomplished by re-cloning the hairpins from passage three into the original viral genome, thereby ensuring no other mutations were present for the final passage. Upon re-administering these three stains to naïve mice, we found they maintained the same population dynamic, suggesting fitness enhancement correlated with amiRNA expression rather than unrelated genetic mutations (Fig. 10A). Additionally, we determined optimal selection with three passages, as clear enrichment trends could be observed at this point. To further corroborate this idea, we again performed a genetic reset and generated a recombinant SINV expressing the most enriched amiRNA identified in this small screen (predicted to target OAS, a

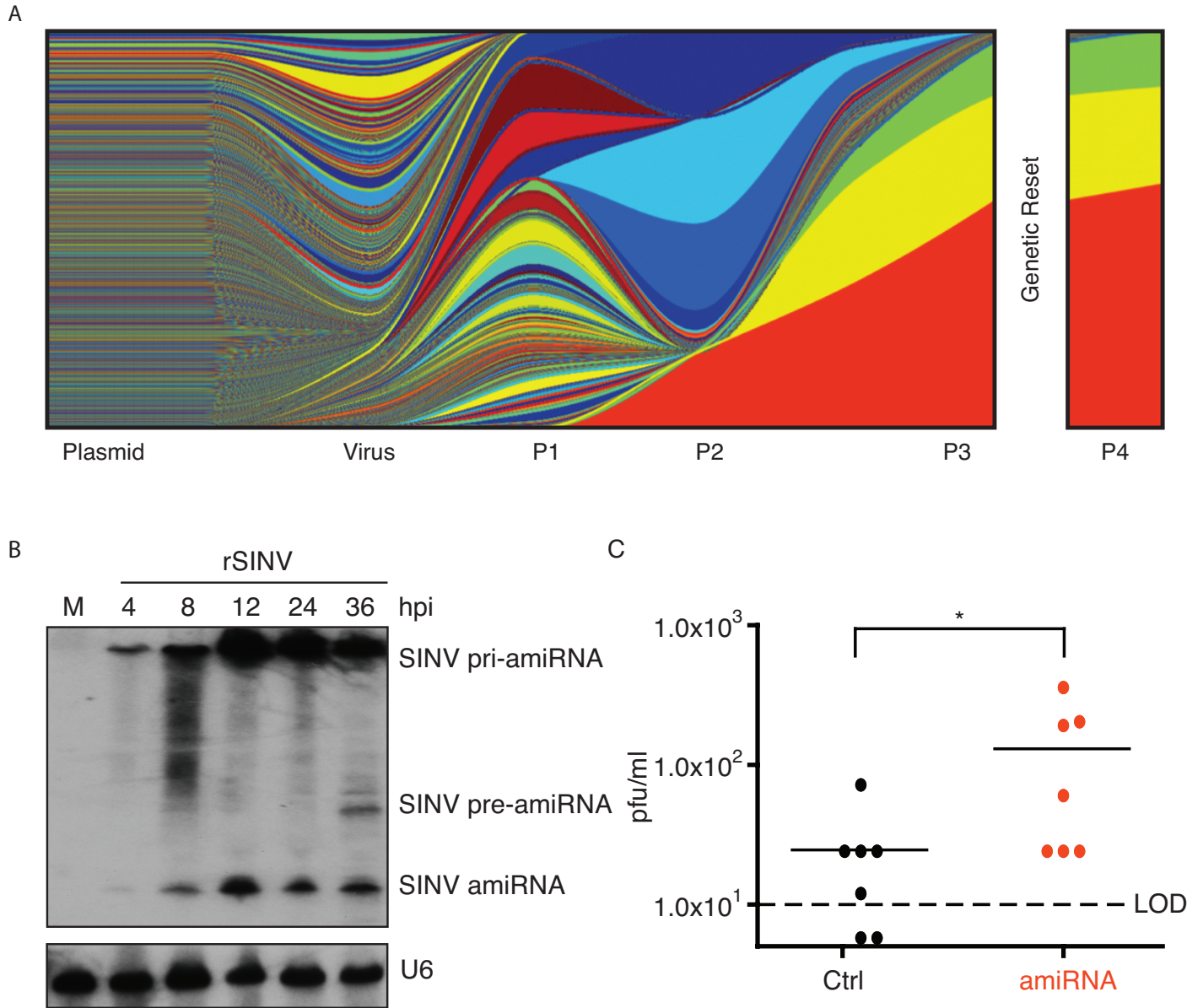


Figure Ten. (A) Matlab plot representing amiRNA viral hairpins present in the overall viral or plasmid populations. Each color depicts a unique amiRNA whose relative proportion corresponds to its abundance in the virus population at indicated passages (P). 'Plasmid' depicts the hairpin diversity present in the SINV cDNA clones, 'Virus' depicts hairpins present in the SINV amiRNA library, and 'P1-P4' depicts hairpins identified in the spleen following each *in vivo* passage. Mice were infected with 5×10^6 plaque forming units (pfu) via footpad injection. (B) Small RNA northern blot derived from cells infected with SINV (MOI=1.0) encoding the most enriched amiRNA from (A). Blot analyzed for amiRNA and U6 expression. (C) Virus titers derived from the spleens of mice infected via footpad injection with 5×10^5 pfu of either SINV-Ctrl or SINV-amiRNA and collected 48hpi. One-tailed Mann-Whitney U-test was used to calculate *P* value, (n=7) **P*<0.05. LOD=limit of detection.

known ISG). Northern blot of cells infected with the recombinant virus demonstrated accurate processing and robust expression of this small RNA (Fig. 10B). Furthermore, we compared virus growth of this amiRNA-containing-virus to a SINV encoding a non-specific amiRNA (Fig. 10C). As expected, incorporation of the amiRNA resulted in titers that were approximately one log higher than the control virus at 48 hrs post *in vivo* infection (Fig. 10C). Taken together, these data demonstrate the *in vivo* efficacy and potential RNA-based VLVs can harness.

Future Directions: Given the silencing potential of these VLV vectors, both *in vitro* and *in vivo*, we also proposed to generate amiRNAs targeting pathogens in an effort to develop vectors capable of therapeutic siRNA delivery. As it turns out, this aspect of the work was too ambitious for the initial scope of this grant and will require additional time and funds to parse out the true potential of this idea. We did attempt to make such a therapeutic (a SINV-based VLV that generated an siRNA against influenza A virus) but we observed no efficacy (data not shown). It is our belief that this pilot study failed because we were unable to delivery the antiviral siRNA to the infected cells in a timely fashion – a result which was likely caused by viral co-infection interference. While we genuinely believe that this work should be pursued further, as a final report for this grant proposal, this is the only component that remains unsuccessful.

Concluding Statement: With the support of this grant, we were able to illustrate that a self-replicating, non-infectious RNA, modeled on either nuclear or cytoplasmic-based viruses, provides a versatile delivery system for silencing and/or expressing a desired RNA for therapeutic purposes.

References:

- Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281-297.
- Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J., and Elledge, S.J. (2008). Identification of host proteins required for HIV infection through a functional genomic screen. *Science* 319, 921-926.
- Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V.B., Wong, E., Orlov, Y.L., Zhang, W., Jiang, J., *et al.* (2008). Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* 133, 1106-1117.
- Cheng, E.H., Levine, B., Boise, L.H., Thompson, C.B., and Hardwick, J.M. (1996). Bax-independent inhibition of apoptosis by Bcl-XL. *Nature* 379, 554-556.
- Cullen, B.R. (2011). Viruses and microRNAs: RISCy interactions with serious consequences. *Genes Dev* 25, 1881-1894.
- Everitt, A.R., Clare, S., Pertel, T., John, S.P., Wash, R.S., Smith, S.E., Chin, C.R., Feeley, E.M., Sims, J.S., Adams, D.J., *et al.* (2012). IFITM3 restricts the morbidity and mortality associated with influenza. *Nature* 484, 519-523.
- Fellmann, C., Zuber, J., McJunkin, K., Chang, K., Malone, C.D., Dickins, R.A., Xu, Q., Hengartner, M.O., Elledge, S.J., Hannon, G.J., *et al.* (2001). Functional identification of optimized RNAi triggers using a massively parallel sensor assay. *Mol Cell* 41, 733-746.
- Ferris, M.T., Aylor, D.L., Bottomly, D., Whitmore, A.C., Aicher, L.D., Bell, T.A., Bradel-Tretheway, B., Bryan, J.T., Buus, R.J., Gralinski, L.E., *et al.* (2013). Modeling host genetic regulation of influenza pathogenesis in the collaborative cross. *PLoS Pathog* 9, e1003196.

- Frolova, E.I., Fayzulin, R.Z., Cook, S.H., Griffin, D.E., Rice, C.M., and Frolov, I. (2002). Roles of nonstructural protein nsP2 and Alpha/Beta interferons in determining the outcome of Sindbis virus infection. *J Virol* 76, 11254-11264.
- Galan-Caridad, J.M., Harel, S., Arenzana, T.L., Hou, Z.E., Doetsch, F.K., Mirny, L.A., and Reizis, B. (2007). Zfx controls the self-renewal of embryonic and hematopoietic stem cells. *Cell* 129, 345-357.
- Garcia-Sastre, A., Egorov, A., Matasov, D., Brandt, S., Levy, D.E., Durbin, J.E., Palese, P., and Muster, T. (1998). Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252, 324-330.
- Griffin, D.E. (2003). Immune responses to RNA-virus infections of the CNS. *Nat Rev Immunol* 3, 493-502.
- Harfe, B.D., McManus, M.T., Mansfield, J.H., Hornstein, E., and Tabin, C.J. (2005). The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci U S A* 102, 10898-10903.
- Hu, G., Kim, J., Xu, Q., Leng, Y., Orkin, S.H., and Elledge, S.J. (2009). A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. *Genes Dev* 23, 837-848.
- Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37, 1-13.
- Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4, 44-57.
- Hurlin, P.J., Steingrimsson, E., Copeland, N.G., Jenkins, N.A., and Eisenman, R.N. (1999). Mga, a dual-specificity transcription factor that interacts with Max and contains a T-domain DNA-binding motif. *EMBO J* 18, 7019-7028.
- Kincaid, R.P., Burke, J.M., and Sullivan, C.S. (2012). RNA virus microRNA that mimics a B-cell oncomiR. *Proc Natl Acad Sci U S A* 109, 3077-3082.
- Kolakofsky, D., Kowalinski, E., and Cusack, S. (2012). A structure-based model of RIG-I activation. *RNA* 18, 2118-2127.
- Konig, R., Stertz, S., Zhou, Y., Inoue, A., Hoffmann, H.H., Bhattacharyya, S., Alamares, J.G., Tscherne, D.M., Ortigoza, M.B., Liang, Y., *et al.* (2009). Human host factors required for influenza virus replication. *Nature* 463, 813-817.
- Konig, R., Zhou, Y., Elleder, D., Diamond, T.L., Bonamy, G.M., Irelan, J.T., Chiang, C.Y., Tu, B.P., De Jesus, P.D., Lilley, C.E., *et al.* (2008). Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell* 135, 49-60.
- Krishnan, M.N., Ng, A., Sukumaran, B., Gilfoy, F.D., Uchil, P.D., Sultana, H., Brass, A.L., Adametz, R., Tsui, M., Qian, F., *et al.* (2008). RNA interference screen for human genes associated with West Nile virus infection. *Nature* 455, 242-245.
- Langlois, R.A., Shapiro, J.S., Pham, A.M., and tenOever, B.R. (2011). In vivo delivery of cytoplasmic RNA virus-derived miRNAs. *Mol Ther* 20, 367-375.

- Levy, D.E., and Darnell, J.E., Jr. (2002). Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3, 651-662.
- Li, J., Ding, S.C., Cho, H., Chung, B.C., Gale, M., Jr., Chanda, S.K., and Diamond, M.S. (2013). A short hairpin RNA screen of interferon-stimulated genes identifies a novel negative regulator of the cellular antiviral response. *MBio* 4.
- Li, Q., Brass, A.L., Ng, A., Hu, Z., Xavier, R.J., Liang, T.J., and Elledge, S.J. (2009). A genome-wide genetic screen for host factors required for hepatitis C virus propagation. *Proc Natl Acad Sci U S A* 106, 16410-16415.
- Loo, Y.M., and Gale, M., Jr. (2011). Immune signaling by RIG-I-like receptors. *Immunity* 34, 680-692.
- Makeyev, E.V., Zhang, J., Carrasco, M.A., and Maniatis, T. (2007). The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol Cell* 27, 435-448.
- McWhirter, S.M., tenOever, B.R., and Maniatis, T. (2005). Connecting mitochondria and innate immunity. *Cell* 122, 645-647.
- Pall, G.S., and Hamilton, A.J. (2008). Improved northern blot method for enhanced detection of small RNA. *Nat Protoc* 3, 1077-1084.
- Parameswaran, P., Sklan, E., Wilkins, C., Burgon, T., Samuel, M.A., Lu, R., Ansel, K.M., Heissmeyer, V., Einav, S., Jackson, W., *et al.* (2010). Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathog* 6, e1000764.
- Perez, J.T., Pham, A.M., Lorini, M.H., Chua, M.A., Steel, J., and tenOever, B.R. (2009). MicroRNA-mediated species-specific attenuation of influenza A virus. *Nat Biotechnol* 27, 572-576.
- Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F.A., van Dyk, L.F., Ho, C.K., Shuman, S., Chien, M., *et al.* (2005). Identification of microRNAs of the herpesvirus family. *Nat Methods* 2, 269-276.
- Pfeffer, S., Zavolan, M., Grasser, F.A., Chien, M., Russo, J.J., Ju, J., John, B., Enright, A.J., Marks, D., Sander, C., *et al.* (2004). Identification of virus-encoded microRNAs. *Science* 304, 734-736.
- Rouha, H., Thurner, C., and Mandl, C.W. (2010). Functional microRNA generated from a cytoplasmic RNA virus. *Nucleic Acids Res.*
- Russell, S.J., Peng, K.W., and Bell, J.C. (2012). Oncolytic virotherapy. *Nat Biotechnol* 30, 658-670.
- Schilte, C., Couderc, T., Chretien, F., Sourisseau, M., Gangneux, N., Guivel-Benhassine, F., Kraxner, A., Tschopp, J., Higgs, S., Michault, A., *et al.* (2010). Type I IFN controls chikungunya virus via its action on nonhematopoietic cells. *J Exp Med* 207, 429-442.
- Schoggins, J.W., Wilson, S.J., Panis, M., Murphy, M.Y., Jones, C.T., Bieniasz, P., and Rice, C.M. (2011). A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 472, 481-485.

- Sessions, O.M., Barrows, N.J., Souza-Neto, J.A., Robinson, T.J., Hershey, C.L., Rodgers, M.A., Ramirez, J.L., Dimopoulos, G., Yang, P.L., Pearson, J.L., *et al.* (2009). Discovery of insect and human dengue virus host factors. *Nature* *458*, 1047-1050.
- Shapiro, J.S., Langlois, R.A., Pham, A.M., and tenOever, B.R. (2012). Evidence for a cytoplasmic microprocessor of pri-miRNAs. *RNA* *18*, 1338-1346.
- Shapiro, J.S., Varble, A., Pham, A.M., and tenOever, B.R. (2010). Noncanonical cytoplasmic processing of viral microRNAs. *RNA*.
- Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R., *et al.* (2005). Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet* *37*, 1281-1288.
- Takeuchi, O., and Akira, S. (2008). MDA5/RIG-I and virus recognition. *Curr Opin Immunol* *20*, 17-22.
- tenOever, B.R. (2013). RNA viruses and the host microRNA machinery. *Nat Rev Microbiol* *11*, 169-180.
- tenOever, B.R., Ng, S.L., Chua, M.A., McWhirter, S.M., Garcia-Sastre, A., and Maniatis, T. (2007). Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. *Science* *315*, 1274-1278.
- Varble, A., Chua, M.A., Perez, J.T., Manicassamy, B., Garcia-Sastre, A., and tenOever, B.R. (2010). Engineered RNA viral synthesis of microRNAs. *Proc Natl Acad Sci U S A* *107*, 11519-11524.
- Vignuzzi, M., Stone, J.K., Arnold, J.J., Cameron, C.E., and Andino, R. (2006). Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* *439*, 344-348.
- Zeng, Y., Wagner, E.J., and Cullen, B.R. (2002). Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell* *9*, 1327-1333.